THE INTERFERENCE OF STRESS ON PHYSOSTIGMINE PRETREATMENT AGAINST SOMAN INTOXICATION IN GUINEA PIGS

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INTRODUCTION

During research efforts towards finding effective drugs are performed in a standard laboratory situation. However, in a more realistic situation other factors may interfere with the treatment regime. There is growing evidence that stress occurring during military operations can impair the efficacy and appearance of side effects of medical treatment. It is known that stress can change the kinetics of the pretreatment (1) and, therefore, affect the protective ratio and evoke the appearance of side effects. During operation Desert Storm soldiers were given pyridostigmine (PYR) tablets against intoxication with acetylcholinesterase (AChE) inhibitors. The employed dose of PYR was expected not to show undesirable cholinergic effects. Nevertheless, peripheral and central side effects were recorded (2). These effects could be the result of stress. First of all, stress itself could be an important factor. It induces prolonged corticosterone secretion that leads to a reduction of hippocampal corticosteroid receptors, which affects other transmitter systems, such as acetylcholine (3). Secondly, stress enhances the passage across the blood-brain barrier (1). In operation Desert Storm nine cases of PYR self-poisoning were encountered. These individuals only suffered from peripheral cholinergic symptoms, whereas no effects on the central nervous system were observed (4). This supports the idea that a combination of many factors including stress plays a role in the appearance of side effects.

In earlier studies pretreatment with physostigmine (PHY) has proved to be very effective against sarin or soman-intoxication (5). Furthermore, PHY was found to be more effective against soman intoxication than PYR in rats (6,7) and in guinea pigs (8). In the course of these studies it was realised that the protective efficacy and the side effects of the pretreatment should also be examined in stressful situations. Therefore, in tn this study the effects of stress on side effects of PHY (0.025 mg/kg/hr) pretreatment and its efficacy against soman intoxication was determined in guinea pigs. To prevent unwanted side effects due to AChE inhibition PHY the pretreatment was combined with the muscarinic receptor antagonist scopolamine (SCO) (0.018 mg/kg/hr) (9). Stress factors were chosen to represent military conditions: emotional stress, physical stress and psychological stress. Most effects can be expected to be centrally mediated effects that may induce changes in different types of behavior. For this reason behavioral read-out systems were used to elucidate the severity of PHY side effects and soman induced incapacitation.

MATERIALS AND METHODS

Animals: Male Dunkin-Hartley albino guinea pigs CrL:(HA)BR (Charles River) with an initial body weight of 400-450 g were used. The animals were kept singly in a cage (Makrolon type IV). The ambient temperature was 20-22^{o}C. Relative humidity was kept over 50%. Food and water were always available. The experiments received prior approval by an independent ethical committee.

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Drug solutions: Physostigmine (eserine) and scopolamine bromide were obtained from Sigma (St.Louis, U.S.A.). Atropine Sulphate was obtained from ACF (Amsterdam, The Netherlands). Soman (O-pinacolyl methylphosphonofluoridate) was synthesized at the TNO Prins Maurits Laboratory. The employed dose of PHY (0.025 mg/kg/hr) offers the recommended blood-AChE inhibition of about 35 % (8). SCO (0.018 mg/kg/hr for a period of ten days) leads to a SCO plasma concentration of 45 nM (8). This was comparable with the level found after a single SC injection of 0.1 mg/kg SCO (43 nM). This SCO plasma concentration did not lead to side effects on behavior, and could antagonise PHY induced side effects (9, 10). The vehicle consisted of 20% propylene glycol, 10% ethanol and 70% water (0.05 % v/v glacial acetic acid water). The drugs used were solved in the vehicle. Because the animals gain weight during the pretreatment period, the PHY and SCO concentrations were based on the estimated weight of the animals one week after implantation based on the normal growth curve for guinea pigs in our laboratory.

Implantation of osmotic mini-pump: Alzet® Osmotic Mini-pumps with a constant delivery rate of 0.55 µl/hr (Model 2002, Alza Corp., Palo Alto, USA) were used to deliver either the vehicle or drug solution. The pumps were implanted subcutaneously on the backs of the animals under ketamine/ventracil anaesthesia. The wounds were sutured with woundclips.

Study design: The study was performed in two different treatment groups of animals (n= 8 animals/group). Both groups were pretreated with PHY (0.025 mg/kg/hr) and SCO (0.018 mg/kg/hr) during 11 days, intoxicated with 2x LD50 soman at day 11, and after one minute followed by a post intoxication therapy with atropine sulphate (AS) (0.36 mg/kg im). The dose of soman (applied subcutaneously) used was 24.5 µg/kg (11) (1 LD50). The animals of the non-stress group were handled by the standard procedures and the animals of the stress group were exposed to intermittent variable, unpredictable and uncontrollable stress during 8 weeks, consisting of cold stress (30 min in a refrigerator), psychological stress (footshocks with an interval of 10 min), physical stress (swimming task and running wheel) and emotional stress (placing the animal in an unfamiliar territory for 30 min).

After the animals were trained in a conditioned learning task, the shuttlebox, the baseline values of the different read-out systems were collected. The body weight, plasma cortisol level, blood-AChE activity (for testing the efficacy of the osmotic pumps), shuttlebox, startle response, and exploration activity in the Open Field task were determined. Subsequently two matched subgroups of 8 animals each were formed that showed no significant differences in any of the behavioral tests. The animals from the stress group received the daily stress factors (with exception of the weekends). Once a week the shuttlebox performance, the startle response, and the body weight were determined. Every other week the animals were tested in the Open Field task and bloodsamples were collected for measuring the plasma cortisol level. After six weeks of stress induction, Alzet® osmotic mini-pumps, containing PHY and SCO, were implanted in all animals. This was called day 0. During the pretreatment period the animals from the stress group were still exposed to the daily stress occasions. At day eleven of the continuously administered pretreatment all animals were intoxicated with 2x LD50 soman as described above. Afterwards the osmotic pumps were not removed.

The efficacy of the PHY and SCO pretreatment with or without stress treatment in counteracting soman-induced post intoxication incapacitation was investigated by observing the post intoxication symptoms, such as hyper-salivation,

tremors and convulsions immediately after soman intoxication and by measuring behavioral parameters after the intoxication symptoms became less severe. These tests started 2 hours after soman intoxication (day 11) and were repeated at day 12, 13, 14 and 18. In a parallel experiment the brain AChE inhibition was measured after a single subcutaneous injection of PHY (0.3 mg/kg) or soman (16% and 40% of the LD50) in stressed and non-stressed guinea pigs. Stress was induced two days before injection and immediately before injection of the compound. Day one cold stress (30 min in a refrigerator), day two emotional stress (placing the animal in an unfamiliar territory for 30 min), and day three physical stress (swimming task) was induced. 30 minutes after injection the animals were decapitated for the brain AChE activity.

Behavioral tests: Four different behavioral tests werd employed during this study*:*

Shuttlebox test: In this test the active avoidance of an unpleasant event upon a conditioned stimulus is used to measure the retrieval of learned behavior. For this test an automated two-way shuttlebox, consisting of two equal compartments of 23x23x23 cm with rounded corners, connected by a photo-cell-guarded gate, is used. The animals have to learn how to avoid a stream of air (about 6 l/s, air tube diameter 1 cm) aimed at their fur within 10 s after presentation of a sound stimulus. During the daily training and test sessions the animals receive 20 trials at an intertrial interval of 20-30 s (random). Only animals that reaches the criterion of 80% or more correct avoidance reactions (CARs) after training, were used in the experiments (10). The number of CARs was used to express the active avoidance performance.

Open Field test: This technique is used to measure parameters of spontaneous behavior, like locomotor activity and exploration in a quantitative way (12). The test consists of a black field of 100 x 100 cm, with 25 cm high enclosing walls. A black grating covers the top of this box. The test room is homogeneously illuminated (100 lux) with a background noise of 52 dB. A videocamera is placed above the OF for registering the movement patterns of the white animal in the black area during a 10 min session. The moving patterns are downloaded into a computer. The following parameters were studied: 1) the distance run, 2) the time spent in the inner field, i.e. a 60x60 cm virtual area in the center of the field, 3) the number of crossings from outer to inner field, and 4) the number of times the rat changes corners. Corners are defined as virtual squares of 20x20 cm in each corner of the field. All parameters are expressed in a cumulative fashion.

Auditory startle response test: In this test the stretching movement of the hindpaws is used to reflect the reaction of the animal on a startle signal (13). For this test the animals are exposed to 20 auditory startle pulses (120 dB, 10 kHz, 20 ms) while standing with their hindpaws on a platform in a vertically mounted PVC-tube (diameter 7 cm, length 16.5 cm). The startle response of 200 ms duration is measured by a transducer connected with the platform, registering the force exerted by the animal upon presentation of the stimulus. An AD converter of an IBM-compatible PC digitised the responses. The area under the curve (AUC), amplitude and latency of the startle response are registered and used to express the motor reaction of the startle reflex.

Determination of cortisol plasma levels: Cortisol plasma levels were determined using a cortisol-kit of ICN. Blood (about 60 µl) obtained from the ear vein of the guinea pig were mixed with heparin and centrifuged for 8 min at 2000 g. The supernatants were stored at -20^{o}C. Within 10 days the cortisol plasma level was determined in a radio immuno assay. Plasma (25µl) was applied in antibodies coated

tubes followed by 0.5 ml of a solution with ^{125} I-cortisol. Thereafter bound and unbound radioactivity was separated and bound radioactivity was counted after which the cortisol concentration could be calculated.

Determination of AChE-activity: Blood samples (5ìl) were obtained from the ear vein of the guinea pig, immediately mixed with 1% saponin (BDH, Poole, UK), frozen in liquid nitrogen and stored at -70 ^{o}C. After appropiate dilution, AChE-activity was assessed using a radiometric method. The ACh end-concentration used was 12 ìm; [3H]ACh iodide (NEN, Dreicich, Germany) was diluted to a specific activity of 602 MBq.mmol^{-1}. Ethopropazine (2.5 ìM, St. Louis, Mo, USA) was used as a specific inhibitor of butyrylcholinesterase. Electric eel AChE was used as a reference.
After decapitation the brain (cerebrum) was quickly isolated, weighed and homogenized (1:10, w/v) in 50 mM Tris/HCL (Ph 7.4), 1 M NaCl, 5 mm EDTA and 1% Triton X-100, using a Braun Melsungen Potter-Elvejhem type homogenizer (Melsungen, Germany). Homogenates were centrifuged for 10 min at 3000 g and the supernatants were kept in liquid N_2 until determination of AChE-activity was carried out as mentioned above.

Statistics: For statistical analysis of the behavioral tests an analysis of variance (two-way ANOVA) was used. For the symptomatology after soman intoxication a Fisher exact probability test or an unpaired t-test with Welch's correction was used. In all tests p values < 0.05 were considered significant.

RESULTS

In this study the effect of stress alone, on the appearance of side effects during PHY and SCO pretreatment, and on the efficacy of the pretreatment in preventing the toxic influences of 2x LD50 soman was tested.

Effect of the stress procedure alone:

Plasma cortisol levels were measured every two weeks. Blood samples were collected before stress induction, 15 min and 60 min after stress induction (see Fig. 1). The intermittent variable, unpredictable and uncontrollable stress used in this study, induced a strong increase of the plasma cortisol levels measured after 15 and 60 min after the stress induction (at both time-points $p < 0.05$). There was no difference found between 15 and 60 min after stress induction on the increase of plasma cortisol. During the first three weeks of intermittent variable, unpredictable and uncontrollable stress all animals from the stress group showed a significant higher number of intertrial response (ITR) in the shuttlebox (ITR non-stress group: 2.2 ± 0.3, ITR stress group: 7.6 ± 0.9; $p < 0.05$). However, after the first three weeks of stress induction the stressed animals reacted similar to the non-stress animals in the shuttle box. The activity in the Open Field test, on the other hand, was not affected (see also Fig 2). On the startle response a tendency towards an increase of the startle response (amplitude and AUC) was found (see also Fig 3). This effect was not found to be significant. Stress had no effect on the brain AChE inhibition after a single injection in a parallel group of animals (Fig. 4).

Effect of stress on side effects of PHY and SCO pretreatment:

During the 11 days of continuously applied pretreatment of PHY and SCO no effect was found in all test systems used under the standard conditions. Under the stressful conditions, on the other hand, an increase was found in the Open Field test:

the entries corners and inner field were significantly increased (Fig 2; p=0.028 and p=0.022 resp.).

Effect of stress on the efficacy of PHY and SCO pretreatment against 2x LD50 soman:

All animals of both groups (stress and non-stress) survived the 24 and 48 h criteria after intoxication with 2x LD50 soman. Four days after soman (day 15) one animal of the non-stress group died and one day later (day 16) one animal from the stress group died.

The post-intoxication symptoms observed following soman intoxication are summarised in Table 1. The appearance of symptoms is significantly different between stress versus non-stress (two-way ANOVA, $p = 0.004$)

TABLE 1. Post-intoxication symptomatology after 2x LD50 soman and AS in continuously PHY and SCO pretreated guinea pigs under standard conditions (non-stressed) or stress-full conditions (stressed) expressed as the % of the total scoring.

non-stress	animal number									
symptoms	5	9	10	12	13	14	20	21	total animal	severity: % of scorings
chewing	8.2	20.0	6.9	20.7	12.9	7.5	0	0	6/8	12.7 ± 2.6
hypersalivation	0	0	0	0	0	0	0	0	0/8	
mild tremors	25	54.3	72.4	10.3	54.8	38.5	23.1	54.5	8/8	41.6 ± 17.4
severe tremor	0	28.7	17.2	24.1	38.7	30.8	0	13.6	6/8	25.5 ± 3.8
convulsions	0	0	0	62.1	12.9	11.5	0	0	3/8	28.8 ± 16.6
dyspnoea	0	0	0	0	0	0	0	0	0/8	
total symptoms	2	3	3	4	4	4	1	2		2.9 ± 0.4

stress	animal number									
symptoms	3	6	8	15	16	17	19	22	total animal	Severity: % of scorings
chewing	12.2	57.9	69.7	53.6	31.4	51.9	28.6	63.6	8/8	16.3 ± 4.0
hypersalivation	0	0	0	0	14.3	0	17.9	0	2/8	16.1 ± 1.8
mild tremors	65.0	52.6	48.5	42.8	22.9	42.9	21.4	50.0	8/8	43.3 ± 5.2
severe tremor	40.8	34.4	18.2	35.7	31.4	29.5	17.9	31.8	8/8	30.0 ± 2.9
convulsions	30.6	0	0	13.9	37.1	11.1	35.7	0	5/8	25.7 ± 5.5
dyspnoea	4.1	0	0	0	40.0	0	3.6	0	3/8	15.9 ± 12.1
total symptoms	5	3	3	4	6	4	6	3		4.3 ± 0.5

Total animal: number of animals in which the symptom was observed.
Total symptoms: total number of different symptoms observed in the animal.
Severity of the symptom expressed as the % of total scoring hits of the animals in which the symptom was observed.

All animals of both groups were able to perform the task in the shuttlebox; they showed a normal ITR activity (compartment changes during the inter-trial interval) after soman intoxication. Their performance was significantly decreased from 96.9 ± 1.3 to 28.8 ± 8.8 in the stress group and from 93.8 ± 2.3 to 38.8 ± 8.9 in the non-stress group. No significant difference was found between the two test groups. 24 hours later this effect was slightly improved, but the effect was still present during one week after soman. The effects on the startle response observed after 2x LD50 soman intoxication are shown in Fig. 3. In both test groups an increase of the startle response was observed. This effect was more persistent in the stress group.

DISCUSSION

In this study the effects of exposure to variable, unpredictable and uncontrollable stress on PHY and SCO pretreatment in a therapeutically relevant dose (14), against 2xLD50 soman was tested. This was done by comparing two test groups: stress versus non-stress. All other factors were kept equal. Three aspects were studied: the effects of the stress procedure on the test systems used, the appearance of side effects during pretreatment, and the protection against post-intoxication incapacitation after intoxication by 2x LD50 soman.

The stress procedure did only affect the behavior in the shuttle box during the first three weeks (six weeks in total). The animals showed an increased inter-trial response (ITR: compartment changes during the inter-trial interval). This could be explained as a higher activity of the stressed animals. However, in the Open Field test no increase of the distance run (a measurement of activity) was observed. Presumably the increase of ITR was due of an increased alertness. In case the effect on learning was tested, these animals would learn faster then the non-stressed animals. This is in accordance with the results obtained by Douma et al. (15). They blocked the mineralo-corticoid receptor which displays a high concentration and distinct distribution in the hippocampus, a brain region which is directly involved in the regulation of spatial orientation and learning. This blockade impairs cognitive behavior. However, in our experiment only the effects on learned behavior (memory) was tested. Both groups performed already on their maximum level. This alertness effect was also found on the startle response: a hardly significant increase of the startle reaction was found during the six weeks of stress induction which had disappeared after starting with the PHY and SCO pretreatment.

During the PHY and SCO pretreatment period of 11 days an effect was found in the Open Field test. The performances in the other test systems were not affected. In a previous study the side effects of PHY and SCO were already tested following the same procedures as in the non-stress group. No side effects were observed in the shuttle box, startle response and neurophysiological parameters (9). In former studies guinea pigs in the Open Field test. It was shown that the pretreatment with PHY and SCO also did not affect this task (Fig. 2). Remarkably, the Open Field test seems to be the only task in which side effects were found in the animals of the stress group. The type of effects (increase of “entries corners” and “inner field”) corresponded with an increased activity. This activity which was also found (although not to be significant) in the "distance run" parameter is presumably due to stimulation of cholinergic receptors induced by the increase of ACh induced by AChE-inhibition after PHY and due to the increased release after stress (3).

The high protection which was found in this study is in accordance with our previous study. The addition of SCO to the pretreatment or addition of AS as post intoxication therapy enhances the protection synergistically against soman induced lethality (16). Only one animal of the non-stress group died after four days. This animal did not show the worst symptoms after soman (12.9 % convulsions observations of the total scoring hits; the mean value was 28.8 %). Presumably there were other factors that could play a role in the condition of that animal. It acted quite different in the behavior tasks before any treatment: there was a very high "time spent in inner field" in the OF task, a very small reaction on the startle reflex and a very gradually and late training curve in the shuttlebox performance. The animal died after a period of diarrhea. In the stress group also one animal died after a period of dyspnoea five days after soman. This animal exhibited the worst post-intoxication symptoms. If no AS post intoxication therapy or no SCO was added to the pretreatment the protection in a standard laboratory situation, was not 100% (16). In case only PHY was added to the pretreatment all animals died within 24 hours. Therefore, we have chosen for the complete treatment (PHY, SCO and AS). It could be that the scenario without SCO or AS would show a bigger difference on lethality between stress and non-stress.

That there is a difference between stress and non-stress animals can be concluded from the observations of the post-intoxication symptomatology. All stressed animals showed severe tremors and five of them convulsions instead of 3 in the non-stress group. Dyspnoea was only found in the stressed animals. In a former study effects like dyspnoea were only found in animals without a post-intoxication therapy

with AS (16). Therefore, these effects in a stressful situation could be the results of a combination of an increase of ACh release and AChE-inhibition or the system become more susceptible to ACh. Indeed after inescapable stress the ACh release was significant increased in the hippocampus and prefrontal cortex investigated with microdialysis technique (3), and the maximal number of muscarinic receptors (Bmax) in several brain areas such as the cortical layers, the CA1 field of the hippocampus and caudate-putamen was significantly increased (17). Furthermore, it could be that the blood-brain barrier permeability was increased through which PHY and soman could easier enter into the brain. It seems that already short-lasting immobilization stress shows this effect (18). Even when it is corrected for the decreased cerebral blood flow, a higher penetration into the CNS was found (19). However, no differences were found in brain AChE inhibition between stressed and non-stressed guinea pigs (Fig. 4).

The animals from the non-stress group showed the best protection against the soman induced intoxication incapacitation. After soman a high increase of the startle amplitude and AUC were found in both groups. In the stress group this effect is more persistent: an increase or a tendency towards an increase of the startle response was still found after one week.

In a previous study it was clarified that direct effects on nicotinergic receptors were involved in the effects on the startle amplitude instead of AChE inhibition (20). It is known that soman has besides its AChE-inhibiting effect, like PHY (21, 22), also direct effects on nicotinergic receptors (23). Furthermore, effects on the release of 5-HT after stress may play a role on the startle reflex (13). Serotonine also seems to play a role in the increased permeability of the blood-brain barrier under stress conditions (24).

From the present experiments it can be concluded that stress indeed affects the efficacy of the pretreatment. This can be due to the changes in the blood-brain barrier and other cholinergic effects, such as the increased release of ACh and up-regulation of muscarinic receptors. Although PHY already easily penetrate into the brain because of its structure, stress also influences the appearance of unwanted side effects of the pretreatment. This effect would be worse in case a drug is used that normally hardly enters the brain, especially when this drug is only tested in a standard laboratory situation.

In conclusion, in a more realistic situation stress seems to interfere with the pretreatment against soman intoxication. Stress evokes the appearance of side effects and decreases protection against soman intoxication. Therefore, other risk factors should be incorporated in the experimentation set-up and not be ruled out during research in the area of treatment by medication.

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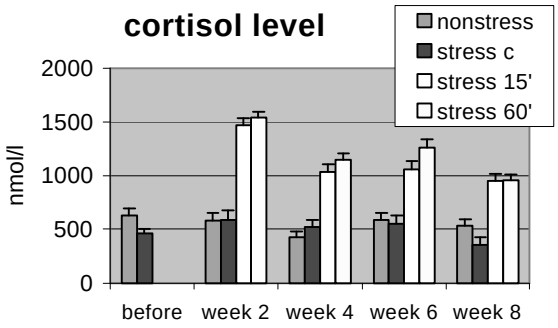

Figure 1. Plasma cortisol levels measured in stressed guinea pigs and in non-stressed guinea pigs after 2, 4, 6, and 8 weeks of stress induction. During week 7 and 8 all animals were also pretreated with PHY and SCO. The cortisol values of the stressed animals were measured before (stress c), 15 min, and 60 min after stress induction. All values after stress induction were significantly increased (ANOVA and Newman-Keuls post-hoc test, $p < 0.05$)..

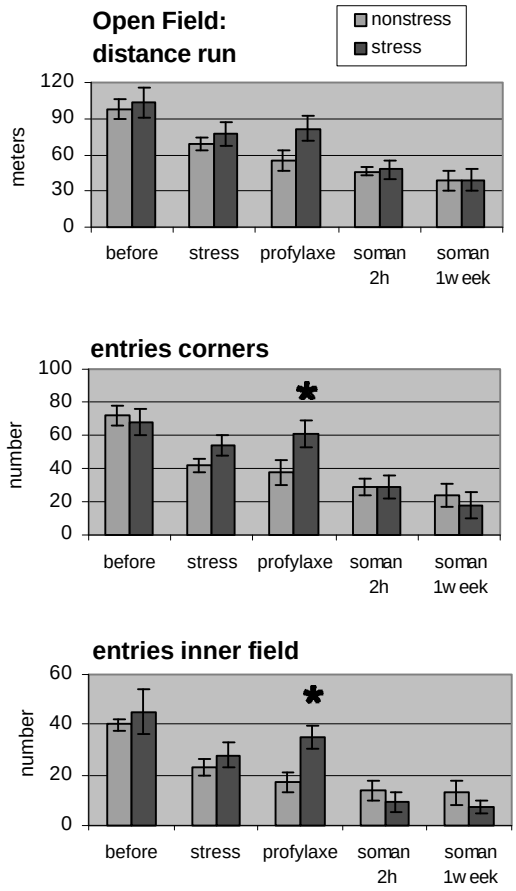

Figure 2. Performance in the OF measured in two different groups (n=8/group), non-stress or stress, before stress induction, after 6 weeks of stress induction (stress group), after 10 days of PHY and SCO pretreatment, two hours and 1 week after 2xLD50 soman. The distance run was expressed as the cumulative meters walked during the 10 min session (mean ± SEM). The entries corners or inner field were expressed as the cumulative number of entries in these virtual areas during the 10 min session (mean ± SEM). * Significantly different from non-stress.

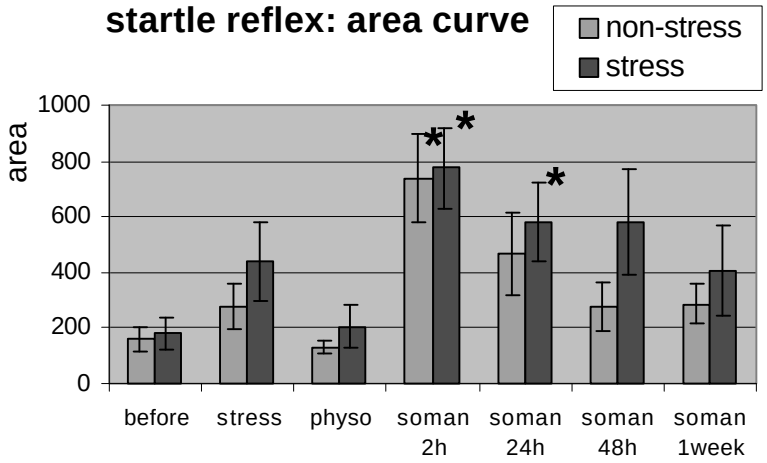

Figure 3. Area of the curve of the startle response of 200 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects before, after 6 weeks of stress induction, after 10 days of PHY/SCO pretreatment and after soman intoxication (2, 24, 48 h and 1 week) in non stressed and stress aguinea pigs (n=8/group, mean ± SEM). * Significantly different from baseline value (before).

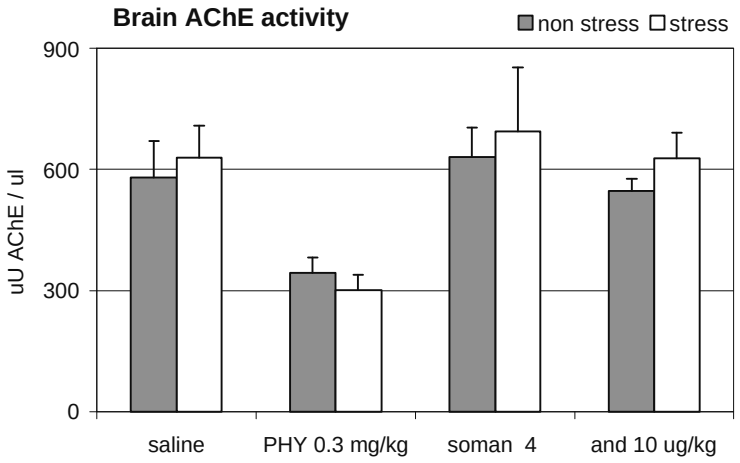

Figure 4. Brain AChE activity (in uU/l) measured in stressed guinea pigs and in non-stressed guinea pigs after 3 days of stress induction. The brain AChE activity was measured on day three, 30 minutes after a subcutaneous injection of saline (n=5) PHY (0.3 mg/kg, n=8), soman (4 ug/kg, n=5 or 10 ug/kg, n=4).